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CDX2 Loss With Microsatellite Stable Phenotype Predicts Poor Clinical Outcome in Stage II Colorectal Carcinoma

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Abstract: Current risk factors in stage II colorectal carcinoma are insufficient to guide treatment decisions. Loss of CDX2 has been shown to associate with poor clinical outcome and predict benefit for adjuvant chemotherapy in stage II and III colorectal carcinoma. The prognostic relevance of CDX2 in stage II disease has not been sufficiently validated, especially in relation to clinical risk factors, such as microsatellite instability (MSI) status, BRAF mutation status, and tumor budding. In this study, we evaluated the protein expression of CDX2 in tumor center and front areas in a tissue microarrays material of stage II colorectal carcinoma patients (n = 232). CDX2 expression showed a partial or total loss in respective areas in 8.6% and 10.9% of patient cases. Patients with loss of CDX2 had shorter disease-specific survival when scored independently either in tumor center or tumor front areas (log rank $P = 0.012$; $P = 0.012$). Loss of CDX2 predicted survival independently of other stage II risk factors, such as MSI status and BRAF mutation status, pT class, and tumor budding (hazard ratio = 5.96, 95% confidence interval = 1.55-22.95; hazard ratio = 3.70, 95% confidence interval = 1.30-10.56). Importantly, CDX2 loss predicted inferior survival only in patients with microsatellite stable, but not with MSI-high phenotype. Interestingly, CDX2 loss associated with low E-cadherin expression, tight junction disruption, and high expression of ezrin protein. The work demonstrates that loss of CDX2 is an independent risk factor of poor disease-specific survival in stage II colorectal carcinoma. Furthermore, the study suggests that CDX2 loss is linked with epithelial-to-mesenchymal transition independently of tumor budding.

Key Words: CDX2, stage II colorectal cancer, microsatellite instability, tumor budding, epithelial-to-mesenchymal transition

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Colorectal carcinoma is one of the most common malignant neoplasms, especially in the countries of the Western world, and its incidence is still increasing.[1] Its prognosis has gradually improved with the development of diagnostics, improved treatment of the disease, and the advent of multidisciplinary teams.[2] In stage I disease, local excision or resection is sufficient treatment, and no adjuvant chemotherapy is needed.[3] In stage III disease, patients clearly benefit from adjuvant treatment, which has been shown to markedly reduce the risk of recurrent disease.[4] In addition, the treatment options for stage IV disease have widened during the last years, especially with the coming of biological treatments, such as anti-EGFR and anti-VEGF treatments, but also by the development of the surgical techniques to remove solitary metastases from the liver and/or lung.[5] However, stage II colorectal carcinoma remains a problem in terms of selecting optimal treatment. Although many risk factors for recurrence in stage II disease have been identified, such as lymphovascular invasion, perineural invasion, preoperative obstruction, preoperative perforation, T4 disease, and <12 lymph nodes examined, a considerable number of patients do suffer relapse without any of these current risk factors.[6] Thus, new biomarkers are needed to identify stage II colorectal carcinoma patients at risk of relapse.

Lately, new promising biomarkers have been identified to risk stratify stage II colorectal carcinoma patients. These include microsatellite instability (MSI) status,[7] tumor budding,[8] immunoscore,[9] and the consensus molecular subtyping,[10] from which the MSI and tumor budding have

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J.S. and T.P. contributed equally.

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been accepted as additional prognostic factors of stage II colorectal carcinoma in TNM8 classification.[11] In our previous studies, we have shown the combined ability of MSI status and ezrin protein expression, and tumor budding grade, to pick up high-risk stage II colorectal carcinoma patients.[12,13] Moreover, several other biomarkers have been documented to better stratify the risk of relapse in stage II colorectal carcinoma.[14] Of them, CDX2 shows up as one of the most promising candidates for further analysis and validation.

CDX2 is a caudal-type homeobox transcription factor, which has a crucial effect on the development, differentiation, and maintenance of colonic epithelium.[15] It orchestrates a gene regulatory network responsible for generating epithelial apical-to-basal polarity in embryonic blastocysts[16,17] and in mouse enterocytes and human colonic epithelial cells.[18] CDX2 has been suggested to possess tumor-suppressing functions by governing several genes responsible for proliferation, migration, and carcinogenesis.[19] CDX2 staining has been used for years in clinical pathology as a marker of intestinal differentiation, and it shows positive nuclear staining in the large majority ($>90\%$ to 100 %) of colorectal and appendix adenocarcinomas.[20] CDX2 expression is often lost in colorectal carcinomas with high tumor grade, advanced tumor stage, BRAF mutation, MSI-high phenotype, and with poor prognosis.[21,22] Recently, the loss of CDX2 protein expression was found to associate with poor prognosis of stage II colon cancer.[23,24] However, the prognostic value of CDX2 loss has not been studied in relation to MSI status, BRAF mutation, and tumor budding in stage II colorectal carcinomas. Furthermore, as CDX2 is important in epithelial cell polarity regulation, and its loss has been suggested to associate with epithelial-to-mesenchymal transition (EMT),[25,26] it would be important to further investigate this connection in colorectal carcinoma progression.

Ezrin, which is an ERM (ezrin-radixin-moesin) family protein, links the plasma membrane and the actin cytoskeleton to organize cellular morphology and cell signaling.[27,28] In normal intestine, ezrin is necessary for maintaining epithelial apical integrity and homeostasis.[29] However, increased ezrin protein expression has been shown to associate with poor outcome in multiple malignancies[30] including colorectal carcinoma.[13,31,32] Frose et al[33] revealed ezrin as an essential protein to mediate EMT and extravasation by rearranging cytoskeleton and cell polarization in breast cancer.

In this work, CDX2 protein expression was studied in a cohort of stage II colorectal carcinoma patients, and CDX2 staining pattern was compared with survival, clinicopathologic variables, MSI status, BRAF mutation status, tumor budding, ezrin expression, and with other markers associated with epithelial junctional polarity and EMT, such as E-cadherin (adherence junctions), Zonula occludens 1 (ZO-1) (tight junctions), integrin beta 4 (ITGB4) (basement membrane), and cytokeratins.

MATERIALS AND METHODS

This study was implemented according to the REMARK guidelines for reporting biomarker studies.[34]

Study Population

We collected archived paraffin-embedded tumor material from consecutive stage II CRC patients operated in Turku University Hospital during the period spanning from 2005 to 2012. This study was approved by the Chief Executive Officer of TYKS-SAPA, Hospital District of Southwest Finland (T52/2014). The use of tissue material was approved by the Scientific Steering Group of Auria Biobank (AB15-8108, 25.5.2012). The study was conducted in accordance with the Declaration of Helsinki. The clinical data were retrieved and histological samples collected and analyzed with the endorsement of the National Authority for Medico-Legal Affairs (VALVIRA, (Dnro 4423/32/300/02). The patient records were accessed anonymously.

During the period spanning from 2005 to 2012, a total of 232 stage II CRC patients were radically operated in our hospital. Computed tomography (CT) of the abdomen and chest x-ray or CT had been performed preoperatively to rule out distant metastases. We carefully checked the patient files, including surgery and pathology reports, and excluded patients with verified lymph node or distant metastases, those who had been operated upon with palliative-intent surgery, and also patients with other than adenocarcinoma histology (eg, neuroendocrine tumors). Only patients with stage II CRC were included in the current study. For tumor staging, TNM7 classification of malignant tumors[35] was used. From the original cohort ($n=232$), for CDX2 evaluation, tumor center spots were available from 209 patients and, for CDX2 staining concerning tumor front spots, from 201 patients. The survival analysis was made from both the tumor center and tumor front spots separately. Of the total 232 stage II colorectal carcinoma patients included in this study, 13 patients with rectal carcinoma had received FU-based preoperative chemotherapy and radiotherapy. Of these patients, CRM was 0.5 mm or less among 7 patients.

Tissue Microarrays Construction

Tissue microarrays (TMA) were constructed and analyzed using the next-generation TMA technique, and the detailed methodology of this technique has been presented in a previous publication.[13] The constructed TMA blocks were sectioned, stained, scanned, and uploaded into the web portal (casecenter.utu.fi), and each individual spot was scored either by a pathologist and senior scientist of biomedicine (K.S., T.P.) or by 2 pathologists (K.S., J.S.). In case of a discrepant result, a consensus score was formed. The resulting scores were combined with the clinical data for statistical analysis.

Immunohistochemistry

Immunohistochemical (IHC) stainings were performed using standard procedures. Shortly, 3.5 μm sections were cut and stained with monoclonal antibodies against MLH1 (Clone G168-15BD Pharmingen, dilution: 1:5), MSH2 (Clone G219-1129, BD Pharmingen, dilution: 1:200), and MSH6 (Clone EP49, Epitomoc, dilution: 1:200). The signal was detected with UltraView Universal DAB Detection kit. For PMS2, Clone EPR3947 (Ventana/Roche, ready to use

antibody) was used, and the signal was detected with OptiView Universal DAB Detection Kit and amplification kit. To detect *BRAF V600E* mutation, BRAF RTU antibody (Clone VE1, Roche/Ventana) was used, and the signal was detected with OptiView Universal DAB Detection kit. For ezrin staining, immunoglobulin G antibody to human ezrin (clone 3C12) was used. For CDX2 staining, CDX2 ready to use antibody (clone EPR2764Y, Diagnostics, Roche) was used. All the stainings were performed with BenchMark XT (Ventana/Roche) using ultraVIEW Universal DAB Detection Kit (Ventana/Roche), except for ezrin, which was carried out with LabVision immunoautomate (Thermo Fisher Scientific) using the Power Vision Plus poly HRP antimouse/rabbit/rat IgG detection kit.

For the epithelial polarity and EMT markers, a multiplex IHC was performed, as described in a previous study.[12] The following antibodies were used. A combination of PanCk clones (C-11, ab7753, Abcam: 1 to 1500 and AE1/3, MA5-13156, Thermo Fisher Scientific Invitrogen: 1 to 1000), ZO-1 (CST, D7D12; 1 to 500), ITGB4 (CST D8P63: 1 to 100), and ECADH (BD clone 36: 1 to 200) was used. The nuclei were stained using DAPI (Roche, 5 μg/mL). ProLong Gold (Thermo Fisher Scientific) was applied for coverslip mounting.

Evaluation of IHC Stainings

All IHC stainings were separately evaluated by 2 observers (K.S. and J.S. or K.S. and T.P.), blinded to clinical data. CDX2 was evaluated, as it was in Dalerba et al,[23] in the following manner: loss of CDX2 equaled to total loss (initial score 0) or weak/scattered staining in the minority of cells (initial score 1). Conventional staining was given an initial score of 2. Only nuclear staining was counted, and a score per patient tumor center and tumor front was formed. As each patient had 2 replicate TMA cores per tumor center and tumor front, a final score of the replicates was counted by rounding the mean of replicates down as follows: 0 to 0.5 = 0 (negative), 1 to 1.5 = 1 (weak), and 2 (conventional). For final scores, 2 categories were used: negative/weak (CDX2 loss) and conventional CDX2. Normal colonic mucosa TMA spots were used as positive controls. MSI and BRAF V600E mutation status and ezrin expression scores are from our previous publication.[13] Digital image analysis for E-cadherin, ZO-1, ITGB4, and PanCk expression was carried out with CellProfiler 2.2.0. Each marker's mean intensity (expression) was measured within the epithelium compartment, and a mean of tumor center and tumor front spots was counted as described.[12] For final 2-tier scores, a dichotomization was carried out on the basis of a median bin for each marker. The analysis of tumor budding from HE-stained histologic whole sections was performed according to the standards set by the International Tumour Budding Consensus Conference (ITBCC) 2016 and has been previously described.[12]

Statistical Analysis

For the association analyses, the 2-sided Fisher exact or χ^2 association test (χ^2) was used as appropriate. Association analyses were carried out using IBM SPSS 24 (SPSS Inc., Armonk, NY). The Cox proportional hazard regression model and the Kaplan-Meier analysis with log-rank test for survival analysis were performed using R version 3.4.3 (Foundation for Statistical Computing, Vienna, Austria) and RStudio 1.1.383 (RStudio Inc., Boston, MA) with *survival* package 2.41-3. Proportional hazard assumption was tested for each variable using Schoenfeld test. The *P*-values in Cox regression analysis were calculated using a Wald test.

RESULTS

CDX2 Association With Clinicopathologic Variables and Survival

Clinicopathologic characteristics of the study cases are summarized in Table 1. CDX2 nuclear staining from tumor center spots was evaluable from 209 patients. Of them, 18 (8.6 %) patients had a negative or weak/scattered staining (loss of CDX2). Of tumor front spots, 22 (10.9 %) of the evaluable 201 patient cases had a loss of CDX2 staining. There was a high correlation of CDX2 staining between tumor center and tumor front cores across all the patients (Spearman $\rho = 0.809$; $P = 3.35E{-}47$). Altogether, 7 patients had a different staining pattern with respect to center and front cores. Representative images of CDX2

TABLE 1. Patient Characteristics

Number of patients	232		
5-y OS	80.10%		
5-y DFS	86.90%		
5-y DSS	91.10%		
Age (y)		Radicality	
Median	74	R0	214
Range	34-96	R1	15
≤70	92	R2	3
>70	140	Ln count	
Sex		≥12 LNs collected	185
Female	117	<12 LNs collected	47
Male	115	Vascular invasion	
Tumor side		No	179
Right side	112	Yes	39
Left side	120	ND	14
pT status		Adjuvant chemo	
T3N0	190	No	163
T4aN0	21	Yes	68
T4bN0	21	ND	1
Grade		MSI status	
G1	26	MSS	171
G2	154	MSI high	43
G3	51	ND	18
ND	1	BRAF status	
Histology		WT	183
Conventional	205	V600E	28
Mucinous	26	ND	21
ND	1	Neoadjuvant chemo+RT	
Preoperative obstruction		No	205
No	196	Yes	13
Yes	36	ND	14
Tumor perforation			
No	212		
Yes	19		
ND	1		

Chemo indicates chemotherapy; ND, not determined; RT, radiotherapy (rectal carcinoma only).

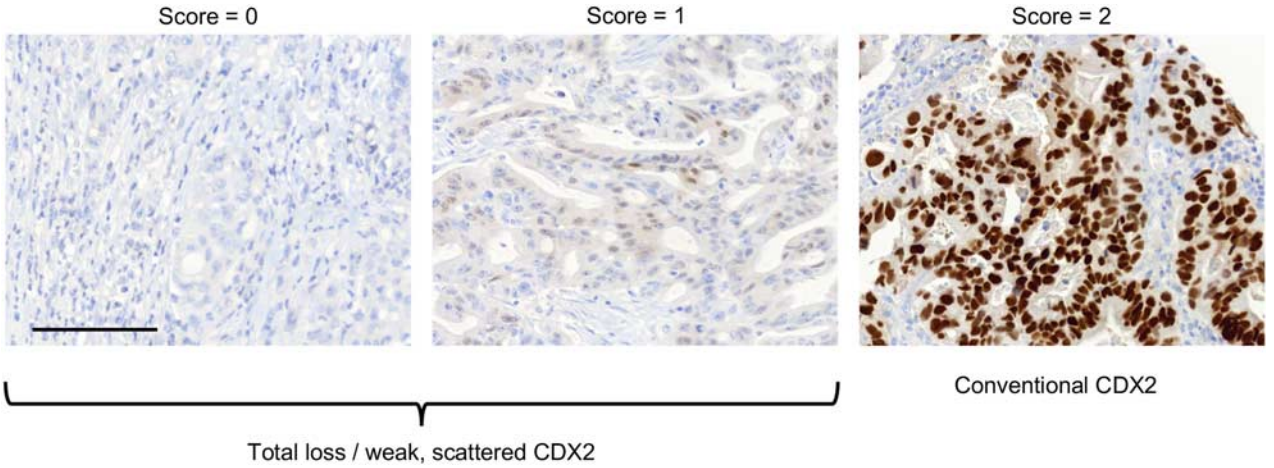

FIGURE 1. Examples of CDX2 staining and scoring. CDX2 was visually evaluated with scores of 0, 1, and 2, representing total loss, weak/scattered staining in minority of cells, and conventional staining, respectively. For subsequent analyses, patients were dichotomized to CDX2 loss group (scores 0 and 1) and to conventional CDX2 group (score 2). Bar, 50 μm.

IHC are shown in Figure 1. Of the clinicopathologic variables, tumor perforation and right-sided tumor correlated with CDX2 loss (Table 2). Loss of CDX2 was associated with shorter disease-free survival (DFS) and disease-specific survival (DSS). None of the other clinicopathologic variables (sex, age, pT status, tumor grade, histologic type, vascular invasion, lymph node count, radicality, preoperative obstruction, adjuvant chemotherapy, or neoadjuvant chemoradiotherapy) correlated with CDX2 staining.

CDX2 Staining in Relation to Microsatellite Stability and BRAF Mutation Status

The majority of patients with CDX2 loss in the tumor center or tumor front had MSI-high phenotype (76% and 64%), while a minority of patients with conventional CDX2 staining were MSI high (15% and 16%). Among MSI-high tumors, 13 of 42 (31%) had a loss of CDX2 in the tumor center, while only 4 of 165 microsatellite stable (MSS) tumors (2.4%) had a loss of CDX2. The results demonstrate that CDX2 loss strongly associates with MSI-high phenotype ($P < 0.001$, Fisher exact test, Table 2).

BRAF mutation status correlated with CDX2 staining, although not as strongly as MSI status. The frequency of BRAF-mutated tumors was 33% and 36% among patients with CDX2 loss in the center or front, respectively, while the mutation was present in 11% and 10% of patients with conventional CDX2 staining in tumor center or front, respectively. Among 26 BRAF-mutated tumors, 8 (31%) were CDX2 low, while, among 172 BRAF WT tumors, 14 (8 %) were CDX2 low in the tumor front cores ($P = 0.003$, Fisher exact test, Table 2).

CDX2 Association With Markers of Epithelial Polarity and EMT

Next, we investigated whether the loss of CDX2 associates with markers of epithelial integrity and polarity. Loss of epithelial junctional and basement membrane integrity, loss of apical-to-basal polarity, and tumor budding are associated with EMT.[36,37] In addition, ezrin, which is a linker of the plasma membrane and cortical actin cytoskeleton, has been recently implicated in the promotion of metastasis through EMT.[33] We found that CDX2 loss in both tumor center and tumor front was associated with loss of E-cadherin, but not with loss of other epithelial marker expressions (ZO-1, ITGB4, PanCk), nor with tumor budding (Table 2). Consistent with the E-cadherin association, CDX2 loss was also associated with loss of tight junction integrity, as measured by ZO-1 perimeter (tight junction perimeter) (Table 2). Interestingly, moderate to strong ezrin protein expression was present in 94% and 95% of patients with CDX2 loss in tumor center or tumor front cores, respectively. Conversely, only one patient (1%) with CDX2 loss had low ezrin protein expression ($P < 0.001$, Pearson χ^2 test). Of all the markers or clinicopathologic variables, the association of CDX2 loss with high ezrin expression was the most significant.

CDX2 Loss as a Predictor of Poor Survival

Given the association of CDX2 loss with DFS and DSS, we next performed prognosis analysis comparing patient groups with conventional CDX2 staining and loss of CDX2. Kaplan-Meier plots demonstrated that CDX2 loss was associated with worse DSS when measured either in the tumor center or tumor front TMA cores ($P = 0.012$; $P = 0.012$) (Fig. 2). The prognostic effect was significant also when measuring DFS as the endpoint ($P = 0.004$; $P = 0.005$). Of the MSS tumor patients with evaluable CDX2 (n = 165), 50% of the patients with CDX2 loss in either tumor center or tumor front died of CRC (4/8 patients). Of the MSI-high tumor patients, only 13% of the patients with CDX2 loss died of CRC (2/15 patients). Accordingly, loss of CDX2 predicted DSS and DFS only in the MSS patient group ($P < 0.001$; $P = 0.019$), but not in the MSI-high group ($P = 0.21$; $P = 0.14$). Examples of immunohistochemical staining patterns from a patient with poor prognosis (loss of CDX2, MSS, BRAFmut,

TABLE 2. Association of CDX2 Expression (Center and Front) With Clinicopathologic Variables, Survival, and Markers Related to Epithelial Integrity and EMT

	CDX2 Expression						
	Center (N = 209), n (%)			Front (N = 201), n (%)			Cox Regression
Variable	Low	High	Fisher Exact (*P*)	Low	High	Fisher Exact (*P*)	Univariate HR (95% CI)
Age (y)			1			1	
≤70 (n = 85)	7 (3)	74 (36)		8 (4)	69 (34)		1 (ref)
>70 (n = 135)	11 (5)	117 (56)		14 (7)	110 (55)		0.82 (0.39-1.75)
Sex			0.628			0.824	
Female (n = 113)	8 (4)	98 (47)		12 (6)	92 (46)		1 (ref)
Male (n = 107)	10 (5)	93 (44)		10 (5)	87 (43)		0.89 (0.42-1.91)
Tumor side			**0.012**			**0.024**	
Right (n = 108)	14 (7)	87 (41)		16 (8)	83 (41)		1 (ref)
Left (n = 112)	4 (2)	104 (50)		4 (3)	96 (48)		1.42 (0.66-3.05)
pT status			0.094			**0.037**	
T3N0 (n = 178)	12 (6)	161 (77)		14 (7)	150 (75)		1 (ref)
T4abN0 (n = 42)	6 (3)	30 (14)		8 (4)	29 (14)		**3.02 (1.38-6.62)**
Grade			0.134			0.179	
G1-2 (n = 169)	11 (6)	151 (72)		14 (7)	140 (70)		1 (ref)
G3 (n = 50)	7 (3)	40 (19)		8 (4)	39 (19)		1.00 (0.40-2.47)
Histology			0.701			0.732	
Conventional (n = 193)	17 (8)	168 (80)		19 (9)	158 (79)		1 (ref)
Mucinous (n = 26)	1 (1)	23 (11)		3 (2)	21 (10)		0.69 (0.16-2.91)
Preoperativeobstruction			0.286			1	
No (n = 186)	14 (7)	166 (79)		19 (9)	154 (77)		1 (ref)
Yes (n = 34)	4 (2)	25 (12)		3 (2)	25 (12)		1.58 (0.64-3.91)
Perforation			**0.001**			**0.003**	
No (n = 203)	12 (6)	181 (87)		16 (8)	168 (84)		1 (ref)
Yes (n = 18)	6 (3)	9 (4)		6 (3)	10 (5)		**4.39 (1.76-10.95)**
Radicality			0.624			0.655	
R0 (n = 203)	16 (8)	178 (85)		20 (10)	167 (83)		1 (ref)
R1-2 (n = 17)	2 (1)	13 (6)		2 (1)	12 (6)		0.59 (0.08-4.37)
Ln count			0.132			0.577	
≥12 LNs (n = 175)	17 (8)	150 (72)		19 (10)	142 (70)		1 (ref)
<12 LNs (n = 45)	1 (1)	41 (19)		3 (2)	37 (18)		1.57 (0.69-3.60)
Vascular invasion			0.745			0.568	
No (n = 171)	15 (7)	145 (74)		16 (8)	136 (72)		1 (ref)
Yes (n = 37)	2 (1)	35 (18)		5 (3)	32 (17)		2.10 (0.92-4.80)
Adj chemo			0.093			0.322	
No (155)	8 (4)	51 (25)		8 (4)	50 (25)		1 (ref)
Yes (64)	9 (4)	140 (67)		13 (6)	129 (65)		1.57 (0.73-3.38)
MSI status			**2.4E−07**			**4.0E−06**	
MSS (n = 165)	4 (2)	161 (78)		8 (4)	150 (75)		1 (ref)
MSI high (n = 42)	13 (6)	29 (14)		14 (7)	28 (14)		0.52 (0.16-1.75)
Neoadjuvant chemo+RT			0.196			0.205	
No (n = 215)	15 (7)	180 (88)		19 (10)	170 (86)		1 (ref)
Yes (n = 13)	2 (1)	8 (4)		2 (1)	6 (3)		0.61 (0.08-4.50)
BRAF status			**0.014**			**0.003**	
WT (n = 183)	12 (6)	169 (81)		14 (7)	158 (80)		1 (ref)
V600E (n = 28)	6 (3)	20 (10)		8 (4)	18 (9)		0.61 (0.14-2.61)
Tumor budding			0.139			0.313	
Low Bd < 7 (196)	14 (7)	169 (81)		18 (9)	157 (79)		1 (ref)
High Bd ≥ 7 (24)	4 (2)	20 (10)		4 (2)	20 (10)		**7.55 (3.39-16.84)**
DFS			**0.017**			**0.019**	
No event (182)	12 (6)	170 (81)		15 (7)	158 (79)		NA
Event (27)	6 (3)	21 (10)		7 (4)	21 (10)		NA
DSS			**0.033**			**0.033**	
No event (186)	13 (6)	173 (83)		16 (8)	161 (8)		NA
Event (23)	5 (2)	18 (9)		6 (3)	18 (9)		NA
ITGB4			0.081			**0.011**	
Low (n = 110)	5 (2)	100 (48)		5 (2)	95 (47)		1 (ref)
High (n = 106)	12 (6)	91 (44)		17 (9)	83 (42)		1.13 (0.52-2.48)
E-cadherin			**0.040**			**0.040**	
Low (n = 107)	13 (6)	92 (44)		16 (8)	84 (42)		1 (ref)
High (n = 109)	4 (2)	99 (48)		6 (3)	94 (47)		0.96 (0.44-2.10)
PanCk			0.312			0.822	
Low (n = 106)	6 (3)	97 (47)		10 (5)	89 (44)		1 (ref)
High (n = 110)	11 (5)	94 (45)		12 (6)	89 (45)		0.74 (0.33-1.62)

TABLE 2. (*continued*)

	CDX2 Expression						
	Center (N = 209), n (%)			Front (N = 201), n (%)			Cox Regression
Variable	Low	High	Fisher Exact (*P*)	Low	High	Fisher Exact (*P*)	Univariate HR (95% CI)
ZO-1			0.312			0.822	
Low (n = 108)	6 (3)	98 (47)		10 (5)	89 (44)		1 (ref)
High (n = 108)	11 (5)	93 (45)		12 (6)	89 (45)		1.76 (0.79-3.94)
TJ perimeter			**0.023**			**0.023**	
Low (n = 108)	13 (6)	90 (43)		16 (8)	84 (42)		1 (ref)
High (n = 108)	4 (2)	101 (49)		6 (3)	94 (47)		0.94 (0.43-2.07)
Ezrin*			**1.9E−08**			**2.0E−08**	
Low (n = 98)	1 (1)	93 (55)		1 (1)	90 (55)		1 (ref)
Intermediate (n = 36)	2 (1)	35 (21)		5 (3)	30 (18)		1.39 (0.42-4.62)
High (n = 38)	13 (8)	25 (14)		15 (9)	23 (14)		3.19 (1.19-8.54)

P-values are 2-sided exact significances.
N-values in left column refer to the Cox regression analysis.
Bold values mark significance, $P < 0.05$.
For HRs, *P*-values not shown.
*Pearson χ^2 for ezrin instead of Fisher exact.
CI indicates confidence interval; HR, hazard ratio, Cox regression univariate (DSS); NA, not applicable; ref, reference.

strong ezrin) and from a patient with favorable prognosis (conventional CDX2, MSI high, BRAFwt, weak ezrin) are shown in Figure 3. The prognostic significance of CDX2 loss remained after adjusting for other confounding risk factors in a multivariate cox regression model when CDX2 was scored either in the tumor center or tumor front (hazard ratio = 5.96, 95% confidence interval = 1.55-22.95; hazard ratio = 3.70, 95% confidence interval = 1.30-10.56) (Table 3). Risk factors in the model included were depth of invasion (pT3 vs. pT4) of the primary tumor, number of lymph nodes resected at surgery ($\geq$12 vs. <12), tumor side (left vs. right), tumor perforation (no vs. yes), MSI status (MSI vs. MSS), BRAF mutation (no vs. yes), vascular invasion (no vs. yes), and tumor budding (<7 vs. $\geq$7).

DISCUSSION

For over 20 years ago, caudal-type homeobox transcription factor 2 (CDX2) was found with its highest expression confined to the intestinal epithelium.[38] It became a widely used tumor marker of intestinal origin in clinical pathology, especially as part of a marker panel for tumors of unknown primary origin.[39] CDX2 is important for gut development and homeostasis,[40] but it also belongs to tumor suppressor genes.[41] For this reason, it is not surprising that its downregulation is associated with high tumor grade, lymphatic invasion, right-sided location, more advanced stage, and poor prognosis.[21,22,42,43] Recently, the prognostic value of CDX2 loss was demonstrated also in stage II colon cancer patients.[23,24] In the current study, we show poor DSS and DFS of stage II patients with tumors bearing CDX2 loss, and correlation of this staining pattern with MSI-high phenotype, BRAF V600E mutation, right-sided tumor, tumor perforation, low E-cadherin expression, and disruption of tight junctions, and high ezrin protein expression.

The frequency of CDX2 loss in our study was 8.6% and 10.9% in the center and front TMA spots, respectively. This is very close to the previous reports in stage II colorectal carcinomas using IHC as the readout. Dalerba et al[23] showed 13% and Hansen et al[24] 10% frequency of CDX2$^-$/weak cases. We used the same scoring system as in the Dalerba et al study,[23] wherein CDX2 loss is equal to a complete lack of CDX2 expression or faint nuclear expression in a minority of malignant epithelial cells.

In this study, loss of CDX2 proved to be a risk factor for poor DSS, both in univariate and multivariate analysis. Given the relatively small fraction of patients with a loss of CDX2 (around 10%), the study would have benefited from a larger cohort size. However, as an internal validation, the same results were obtained when CDX2 was analyzed separately, either in the tumor center or tumor front cores. The results also demonstrate that the prognostic value of CDX2 loss is independent on its spatial localization, although this should be better validated using whole section analysis. Previously, Dalerba and colleagues demonstrated that the prognostic power of CDX2 loss was independent of other confounding risk factors, which were age, pT status, and grade.[23] Similarly, Hansen et al[24] showed prognostic independence of CDX2 loss from pT status, age, sex, vascular/perineural invasion, and tumor perforation. However, neither of these 2 earlier studies with stage II material included the MSI status, BRAF mutation status, or tumor budding as risk factors in multivariate models. Our study demonstrates that the prognostic value of CDX2 loss is independent also of these aforementioned risk factors in stage II colorectal carcinoma.

We show that CDX2 loss correlated with MSI-high phenotype, which is in concordance with several previous studies.[21,22,24,44] It has been reported, that MSI-high status is linked with favorable prognosis in stage II colorectal carcinoma,[45] which is contradictory, as CDX2 loss also associates with poor prognosis. Hansen et al[24] showed that CDX2 loss predicted clinical outcome in stage II patients with MSI-high status. Here, in this work, we show that

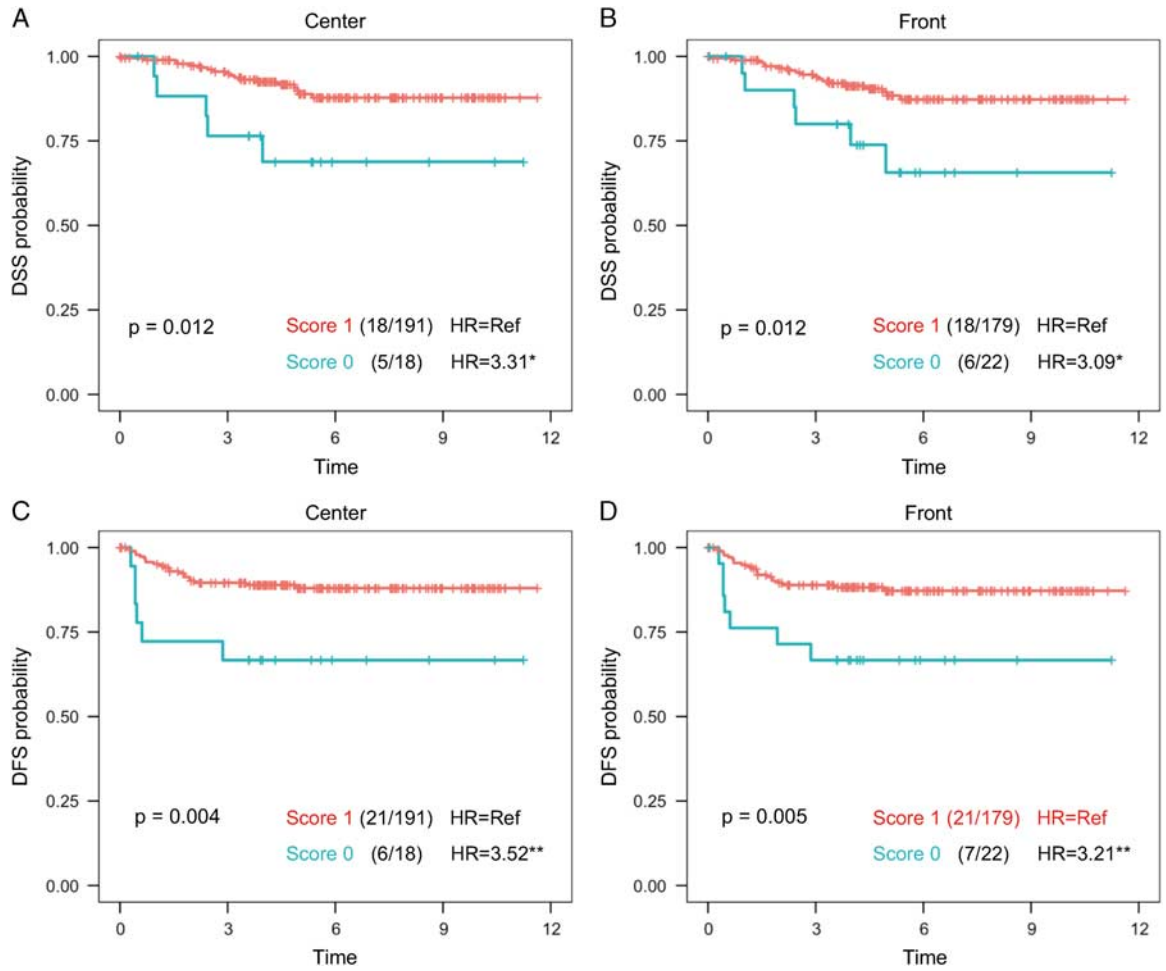

FIGURE 2. Loss of CDX2 predicts survival in stage II colorectal carcinoma. Kaplan-Meier plots with DSS probability (A, B) and DFS probability (C, D) of patients with CDX2 loss (cyan, score 0) and with conventional CDX2 expression (red, score 1). Both the tumor center (A, C) and tumor front cores (B, D) were analyzed separately. *P*-value is based on Log rank test. Values in brackets indicate the number of events and the number of patients within each score group. HR indicates hazard ratio (univariate cox regression) with $*P < 0.05$ and $**P < 0.01$.

CDX2 loss predicted worse outcome (DSS and DFS) within the MSS patient group only, but not when analyzed within the MSI-high patient group. This is in line with recently published work,[46] wherein it was demonstrated that none of the patients with MSI-high and negative CDX2 staining developed distant metastasis, while 75 % of MSS patients with negative CDX2 had a metastasis. Furthermore, Ryan et al[47] showed that loss of CDX2 did not associate with DFS in the mismatch repair–deficient (dMMR = MSI high) group of patients with heterogenous stages (I to IV) (n = 238). Pilati et al[48] further demonstrated that lack of CDX2 was a prognostic factor only in MSS and not in the MSI-high patient group of stage II and III colon cancer. Overall, these results suggest that a combination of MSS phenotype and loss of CDX2 stratifies patients to worse survival outcome than that of MSI-high phenotype and CDX2 loss.

The current work could demonstrate the correlation between BRAF mutation and loss of CDX2, although the correlation was not as strong as with MSI-high phenotype. The association of negative CDX2 staining and BRAF mutation has been reported in earlier publications.[46,49–51] Bruun et al[49] showed that CDX2 loss stratifies stage I to III colorectal patients to poor survival in a BRAF-mutated subgroup. It has been hypothesized that the high prevalence of BRAF mutation in $CDX2^-$ colorectal carcinoma develops through a serrated pathway,[42] although the exact functional mechanism of this correlation remains to be elucidated.

Tumor budding, which is defined by the presence of single carcinoma cells or clusters of up to 4 cells at the invasive front, is a risk factor for poor outcome in stage II colorectal carcinoma.[8,12] Many studies link tumor budding with epithelial-to-mesenchymal transition (EMT).[12,37] In the current study, we found no correlation of CDX2 with respect to tumor budding analyzed from HE-stained whole sections, but we discovered a significant correlation between loss of CDX2 and low E-cadherin,

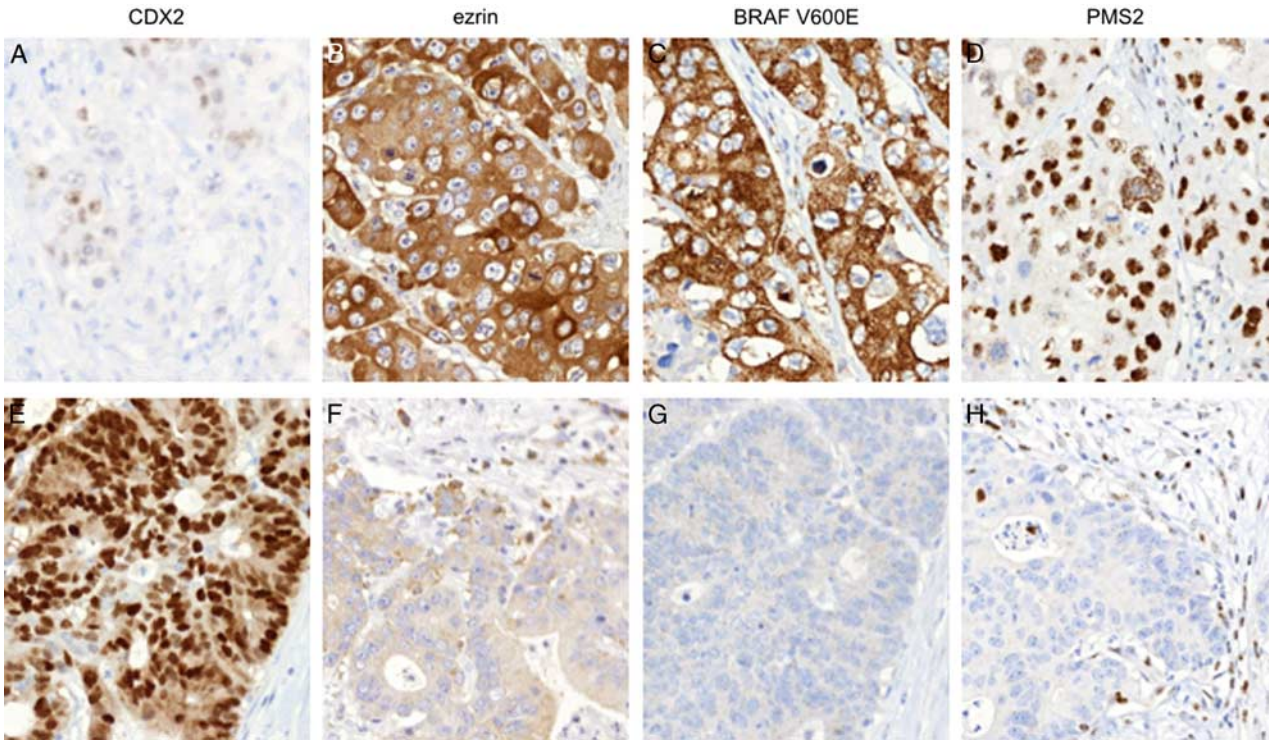

FIGURE 3. Example images of marker expressions in a colorectal carcinoma stage II patient case with poor survival (A–D) and with favorable survival (E–H). The case with poor survival was CDX2 low, ezrin high, BRAF V600E^{+}, and MSS (positive for MLH1, MSH2, MSH6, and PMS2), whereas the case with favorable survival was CDX2 conventional, ezrin low, BRAF wt, and MSI high (MLH1^{-}, MSH2^{+}, MSH6^{+}, and PMS2^{-}).

which is often lost during EMT. Some earlier studies support the relationship between EMT and CDX2 expression. Pilati et al[48] demonstrated that CDX2 loss is prognostic only in a subgroup of patients with consensus molecular subtype 4 (CMS4), which is linked to mesenchymal gene expression and TGFβ pathway activation. Zhang et al[26] demonstrated that forced CDX2 expression induces E-cadherin and reverses EMT in gastric cancer. In another study, it was shown that Claudin-1, which is an important element of tight junctions, whose dysregulation is implicated in EMT, is dependent on CDX2 regulation.[25] In agreement with this study, we show here that CDX2 loss associates with disrupted epithelial tight junctions (ZO-1 perimeter). Overall, these results suggest that loss of CDX2 could functionally associate with EMT, but independently of tumor budding.

Ezrin is a member of the ERM (Ezrin, Radixin, Moesin) complex, which links the actin cytoskeleton to several membrane-associated receptors and adhesion molecules.[28] It plays a role in cell migration, proliferation, survival, and signal transduction including mTOR, Pi3 kinase/Akt, Src, EGFR, Rho-kinase, and protein kinase C pathways[52] and, recently, was shown to mediate EMT and extravasation.[33] For this reason, it is not surprising that its overexpression has been linked with poor outcome in many malignancies including colorectal carcinoma.[13,30–32] In the current study, we found a strong correlation between loss of CDX2 and high ezrin protein expression. According to our knowledge, this association has not been reported before, and it raises the question whether these proteins could regulate each other. Both CDX2 and ezrin play an important role in supporting apical junctional integrity and cell polarity in embryogenesis[16,17] and in normal colonic epithelial cells.[18] Recently, it was shown that depletion of CDX2 resulted in diffuse E-cadherin expression, reduced phosphorylated ezrin (p-ezrin), and disrupted adherence junctions in blastocysts of porcine embryos.[16] Earlier, Gao and Kaestner[18] demonstrated that *Cdx2* knock-out in mouse intestinal epithelium resulted in significant expression changes of 2 different splicing variants of ezrin mRNA. These studies suggest a model whereby CDX2 expression would be upstream of ezrin expression. This could be, however, more complicated, as a mutually reinforcing relationship between epithelial cell polarity and CDX2 expression has been suggested.[17] Further mechanistic studies are needed in order to elaborate on the regulatory relationship of CDX2, ezrin, and cell polarity and how this may affect EMT and metastasis in colorectal carcinoma.

In conclusion, CDX2 loss is quite an infrequent event in stage II colorectal carcinoma, but it is an independent risk factor of poor DSS. The routine analysis of CDX2 staining from stage II colorectal carcinoma patients, along with the determination of MSI status and tumor budding grade, might bring added value to identify high-risk patients in need of adjuvant chemotherapy. However, this would

TABLE 3. Multivariate Cox Regression Analysis of CDX2 and Stage II Risk Factors and Variables With CDX2 Expression Association

Multivariate Cox Regression				
Variable (n Center; n Front)*	**HR Center (95% CI)**	***P***	**HR Front (95% CI)**	***P***
CDX2				
Conventional (n = 191; 179)	1 (ref)		1 (ref)	
Neg/weak (n = 18; 22)	**5.96 (1.55-22.95)**	**0.009**	**3.70 (1.30-10.56)**	**0.014**
Tumor side				
Right (n = 101; 99)	1 (ref)		1 (ref)	
Left (n = 108; 102)	1.30 (0.50-3.00)	0.594	1.25 (0.50-3.12)	0.637
pT status				
T3N0 (n = 173; 164)	1 (ref)		1 (ref)	
T4abN0 (n = 36; 37)	**3.32 (1.34-8.20)**	**0.009**	**2.87 (1.18-6.97)**	**0.02**
Perforation				
No (n = 193; 184)	1 (ref)		1 (ref)	
Yes (n = 15; 16)	**3.62 (1.17-11.3)**	**0.026**	**3.14 (1.03-9.56)**	**0.045**
Ln count				
≥ 12 LNs (n = 175; 161)	1 (ref)		1 (ref)	
< 12 LNs (n = 45; 40)	1.15 (0.40-3.30)	0.795	1.06 (0.38-2.96)	0.914
Vascular invasion				
No (n = 160; 152)	1 (ref)		1 (ref)	
Yes (n = 37; 37)	**3.28 (1.30-8.30)**	**0.012**	2.44 (0.97-6.10)	0.057
MSI status				
MSS (n = 165; 158)	1 (ref)		1 (ref)	
MSI high (n = 42; 41)	0.27 (0.04-1.60)	0.148	0.38 (0.06-2.29)	0.292
BRAF status				
WT (n = 181; 172)	1 (ref)		1 (ref)	
V600E (n = 26; 26)	2.20 (0.34-14.3)	0.41	1.76 (0.23-13.62)	0.586
Tumor budding				
Low Bd < 7 (183; 175)	1 (ref)		1 (ref)	
High Bd ≥ 7 (24; 24)	**3.26 (1.21-8.80)**	**0.02**	**4.11 (1.56-10.82)**	**0.004**

Bold values mark significance, $P < 0.05$ (2-sided, Wald test).

*The numbers in brackets in the variable column indicate patient numbers in the tumor center and tumor front cores, respectively.

CI indicates confidence interval; HR, hazard ratio, Cox regression univariate; ref, reference.

require further validation and consensus of CDX2 scoring criteria. In addition, the relationship of CDX2 loss with EMT-related phenotypic changes (eg, low E-cadherin, junctional disruption, high ezrin) warrants further investigation to find out whether CDX2 is an active regulator or a downstream target of EMT in colorectal carcinoma.

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